APPLICATION

FOR

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TITLE: **APOPTIN-ASSOCIATING PROTEINS**

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Technical Field

The invention relates to the field of apoptosis.

Background

Apoptosis is an active and programmed physiological process for eliminating superfluous, altered or malignant cells (Earnshaw, 1995, Duke et al., 1996). Apoptosis is characterized by shrinkage of cells, segmentation of the nucleus, condensation and cleavage of DNA into domain-sized fragments, in most cells followed by internucleosomal degradation. The apoptotic cells fragment into membrane-enclosed apoptotic bodies. Finally, neighbouring cells and/or macrophages will rapidly phagocytose these dying cells (Wyllie et al., 1980, White, 1996). Cells grown under tissue-culture conditions and cells from tissue material can be analysed for being apoptotic with agents staining DNA, as e.g. DAPI, which stains normal DNA strongly and regularly, whereas apoptotic DNA is stained weakly and/or irregularly (Noteborn et al., 1994, Telford et al., 1992).

The apoptotic process can be initiated by a variety of regulatory stimuli (Wyllie, 1995, White 1996, Levine, 1997). Changes in the cell survival rate play an important role in human pathogenesis of diseases, e.g. in cancer development and auto-immune diseases, where enhanced proliferation or decreased cell death (Kerr et al., 1994, Paulovich, 1997) is observed. A variety of chemotherapeutic compounds and radiation have been demonstrated to induce apoptosis in tumor cells, in many instances via wild-type p53 protein (Thompson, 1995, Bellamy et al., 1995, Steller, 1995, McDonell et al., 1995).

Many tumors, however, acquire a mutation in p53 during their development, often correlating with poor response to cancer therapy. Certain transforming genes of tumorigenic DNA viruses can inactivate p53 by directly binding to it (Teodoro, 1997). An example of such an agent is the large T antigen of the tumor DNA virus SV40. For several (leukemic) tumors, a high expression level of the proto-oncogene Bcl-2 or Bcr-abl is associated with a strong resistance to various apoptosis-inducing chemotherapeutic agents (Hockenberry 1994, Sachs and Lotem, 1997).

For such tumors lacking functional p53 (representing more than half of the tumors) alternative anti-tumor therapies are under development based on induction

of apoptosis independent of p53 (Thompson 1995, Paulovich et al., 1997). One has to search for the factors involved in induction of apoptosis, which do not need p53 and/or can not be blocked by anti-apoptotic activities, such as Bcl-2 or Bcr-abl-like ones. These factors might be part of a distinct apoptosis pathway or might be (far) downstream of the apoptosis inhibiting compounds.

Apoptin is a small protein derived from chicken anemia virus (CAV; Noteborn and De Boer, 1995, Noteborn et al., 1991, Noteborn et al., 1994; 1998a), which can induce apoptosis in human malignant and transformed cell lines, but not in untransformed human cell cultures. In vitro, apoptin fails to induce programmed cell death in normal lymphoid, dermal, epidermal, endothelial and smooth-muscle cells. However, when normal cells are transformed they become susceptible to apoptosis by apoptin. Long-term expression of apoptin in normal human fibroblasts revealed that apoptin has no toxic or transforming activity in these cells (Danen-van Oorschot, 1997 and Noteborn, 1996).

In normal cells, apoptin was found predominantly in the cytoplasm, whereas in transformed or malignant cells i.e. characterized by hyperplasia, metaplasia, dysplasia or aplasia, it was located in the nucleus, suggesting that the localization of apoptin is related to its activity (Danen-van Oorschot et al. 1997).
Apoptin-induced apoptosis occurs in the absence of functional p53 (Zhuang et al., 1995a), and cannot be blocked by Bcl-2, Bcr-abl (Zhuang et al., 1995), or the Bcl-2-associating protein BAG-1 (Danen-Van Oorschot, 1997a, Noteborn, 1996).

Therefore, apoptin is a therapeutic compound for the selective destruction of tumor cells, or other hyperplasia, metaplasia, a- or dysplasia, especially for those tumor cells which have become resistant to (chemo)-therapeutic induction of apoptosis, due to the lack of functional p53 and (over)-expression of Bcl-2 and other apoptosis-inhibiting agents (Noteborn and Pietersen, 1998). It appears, that even pre-malignant, minimally transformed cells, are sensitive to the death-inducing effect of apoptin. In addition, Noteborn and Zhang (1998) have shown that apoptin-induced apoptosis can be used as diagnosis of cancer-prone cells and treatment of cancer-prone cells.

The fact that apoptin does not induce apoptosis in normal human cells, at least not in vitro, shows that a toxic effect of apoptin treatment in vivo will be very

low. Noteborn and Pietersen (1998) and Pietersen et al. (1998) have provided evidence that adenovirus expressed apoptin does not have an acute toxic effect in vivo. In addition, in nude mice it was shown that apoptin has a strong anti-tumor activity.

However, to further enlarge the array of therapeutic anti-cancer or anti-auto-immune-disease compounds available in the art, additional therapeutic compounds are desired that are designed to work alone, sequentially to, or jointly with apoptin, especially in those cases wherein p53 is (partly) non-functional.

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Summary of the Invention

The invention provides novel therapeutic possibilities, for example novel combinatorial therapies or novel therapeutic compounds that can work alone,

sequentially to, or jointly with apoptin, especially in those cases wherein p53 is (partly) non-functional.

In a first embodiment, the invention provides an isolated or recombinant nucleic acid or functional equivalent or fragment thereof encoding an apoptin-associating proteinaceous substance capable of providing apoptosis, alone or in combination with other apoptosis inducing substances, such as apoptin. Proteinaceous substance herein is defined as a substance comprising a peptide, polypeptide or protein, optionally having been modified by for example glycosylation, myristilation, phosporylation, the addition of lipids, by homologous or heterologous di-or multimerisation, or any other (posttranslational) modifications known in the art.

Apoptin-associating herein is defined as belonging to the cascade of substances specifically involved in the cascade of events found in the apoptosis pathway as inducable by apoptin, preferably those substances that are specifically involved in the p53-independent apoptosis pathway.

Brief Description of the Figures

Figure 1 shows the partial sequence of vector pMT2SM-AAP-1-a. The DNA sequence of the AAP-1-a cDNA is given in bold.

Figure 2 shows the partial sequence of vector pMT2SM-AAP-1-b. The DNA sequence of the AAP-1-b cDNA is given in bold.

Figure 3 shows the amino-acid sequence of the analysed region of the apoptin-associating clone AAP-1-b (bold). In addition, the three C-terminal amino acids H-E-G of the multiple cloning site of pACT are given to illustrate that the AAP-1 amino acid sequence is in frame with the GAL4-activation domain. This feature proves that the AAP-1 region is indeed synthesised in yeast cells. Note that in figure 3 amino acid position 23 corresponds with the first amino acid of an AAP-1 like protein. Functional domains or fragments herein can for example be identified as a transcription factor binding domain running from amino acid position 1 (= 23 in figure 3) to about 54; a zinc-finger motive, protein-protein interaction and/or protein-nucleic acid interaction domain running from about amino acid position 25 (= 47 in figure 3) to about 42; an apoptosis associated region running from about

amino acid position 32 to 226; a nuclear localisation signal running from about amino acid position 74 to 81; and a nuclear localisation signal running from about amino acid position 102 to 108, or at equivalent positions in another AAP-1 like protein.

Figure 4 shows the apoptotic activity of AAP-1-b protein in Saos-2 cells, when expressed alone (filled square) or in combination with apoptin (open square). The percentage of apoptin-induced apoptosis is also indicated (filled triangle)

Brief Description of the Preferred Embodiments

In a preferred embodiment, the invention provides an isolated or recombinant nucleic acid or functional equivalent or fragment thereof encoding an apoptin-associating proteinaceous substance capable of providing apoptosis. More preferably the encoded apoptin-associated proteinaceous substance co-localizes with other apoptosis inducing substances, for example apoptin, when the two apoptosis inducing substances are present in the same cell. In normal non-transformed cells the two apoptosis inducing proteins co-localizes in the cytoplasm whereas in transformed or malignant cells the two apoptosis-inducing proteins co-localizes in the nucleus. In another embodiment the apoptin-associating substance is capable of binding to the mouse transcription factor YY1, which was earlier shown to bind to the mouse AAP-1 homologue RYBP (Garcia et al., 1999). In a most preferred embodiment the isolated or recombinant nucleic acid or functional equivalent or fragment thereof encoding an apoptin-associating proteinaceous substance capable of providing apoptosis is derived from a cDNA library, preferably a vertebrate cDNA library, such as derivable from poultry, but more preferably a mammalian cDNA library, preferably wherein said cDNA library comprises human cDNA.

In another embodiment, the invention provides an isolated or recombinant nucleic acid or functional equivalent or fragment thereof encoding an apoptin-associating proteinaceous substance capable of providing apoptosis capable of hybridising to a nucleic acid molecule encoding an apoptin-associating proteinaceous substance capable of providing apoptosis as shown in figure 1 or 2, in particular encoding a novel protein capable of providing apoptosis or functional equivalent or functional fragment thereof called apoptin-associating protein 1,

abbreviated herein also as AAP-1. Of course, an isolated or recombinant nucleic acid or functional equivalent or fragment thereof encoding an additional apoptin-associating proteinaceous substance capable of associating with the AAP-1 protein are herewith also provided, means and methods to arrive at such an additional protein located in the apoptin cascade follow those of the detailed description given herein.

In particular, the invention provides an isolated or recombinant nucleic acid or functional equivalent or fragment thereof encoding an apoptin-associating proteinaceous substance capable of providing apoptosis being at least 70% homologous, preferably at least 80%, more preferably at least 90%, most preferably at least 95% homologous to a nucleic acid molecule, or to a functional equivalent or functional fragment thereof, encoding an apoptin-associating proteinaceous substance as shown in figure 1 or 2.

Furthermore, the invention provides a vector comprising a nucleic acid according to the invention. Examples of such a vector are given in the detailed description given herein; such as vector pMT2SM-AAP-1-a or b, pMT2SM vector expressing Myc-tagged AAP-1-a or AAP-1-b cDNAs, a plasmid expressing an apoptin-associating protein fragment, E.coli overexpression vectors such as pMAL and pET22b comprising a nucleic acid according to the invention and so on. These and other vectors are for example useful in finding additional apoptin-associating proteinaceous substances from the cascade, as defined above, or for the (over)expression of a protein encoded by a nucleic acid according to the invention..

In yet another embodiment, the invention provides a vector comprising a nucleic acid according to the invention, said vector comprising a gene-delivery vehicle, making the invention very useful in gene therapy. By equiping a gene delivery vehicle with a nucleic acid according to the invention, and by targeting said vehicle to a cell or cells that have been over-proliferating and/or have shown decreased death rates, said gene delivery vehicle provides said cell or cells with the necessary means for apoptosis, providing far reaching therapeutic possibilities.

Furthermore, the invention provides a host cell comprising a nucleic acid or a vector according to the invention. Examples comprise transformed or transfected bacterial or yeast cells as described in the detailed description herein. Preferred is a

host cell according to the invention which is a transformed eukaryotic cell such as a yeast cell or a vertebrate cell, such as mammalian or Cos cells transformed or transfected with a nucleic acid or vector according to the invention. Said cells are in general capable to express or produce a proteinaceous substance capable of providing apotosis with the ability to associate with apoptin.

The invention furthermore provides an isolated or recombinant apoptin-associating proteinaceous substance capable of providing apoptosis. As for example shown herein in figure 4, expression of such apoptin-associating proteinaceous substance in cells, such as tumour cells, or other over-proliferating cells, induces the apoptic process. It can do so alone, or in the presence of other apoptosis inducing substances such as apoptin, and especially so independent of p53, showing that also in those cases where (functional) p53 is absent apoptosis can be induced by a substance according to the invention. When the apoptin-associated proteinaceous substance capable of providing apoptosis is used together with another apoptosis inducing substance, for example apoptin, the two proteinaceous substances co-localize in the cytoplasm of normal cells and do not result in apoptosis. Whereas in transformed or malignant cells the two apoptosis-inducing proteins co-localize in the nucleus and induce apoptosis. The invention also provides a proteinaceous substance according to the invention which binds to the transcription factor YY1, which was already shown to bind the AAP1 mouse homologue RYBP. In particular, the invention provides a proteinaceous substance according to the invention encoded by a nucleic acid according to the invention, for example comprising at least a part of an amino acid sequence as shown in figure 3 or a functional equivalent or functional fragment thereof capable of providing apoptosis alone or in combination with other apotosis inducing substances such as apoptin.

The invention also provides an isolated or synthetic antibody specifically recognising a proteinaceous substance or functional equivalent or functional fragment thereof according to the invention. Such an antibody is for example obtainable by immunising an experimental animal with a apoptin-associating proteinaceous substance or an immunogenic fragment or equivalent thereof and harvesting polyclonal antibodies from said immunised animal (as shown herein in the detailed description), or obtainable by other methods known in the art such as

by producing monoclonal antibodies, or (single chain) antibodies or binding proteins expressed from recombinant nucleic acid derived from a nucleic acid library, for example obtainable via phage display techniques.

With such an antibody, the invention also provides a proteinaceous substance specifically recognisable by such an antibody according to the invention, for example obtainable via immunoprecipitation, Western Blotting, or other immunological techniques known in the art.

Furthermore, the invention provides use of a nucleic acid, vector, host cell, or proteinaceous substance according to the invention for the induction of apoptosis, as for example shown in figure 4. In particular, such use is provided wherein said apoptosis is p53-independent. In particular, such use is also provided further comprising use of a nucleic acid encoding apoptin or a functional equivalent or fragment thereof or use of apoptin or a functional equivalent or fragment thereof. As can be seen from figure 4, combining these apoptin-inducing substances increases the percentage apoptosis of treated tumour cells.

Such use as provided by the invention is particularly useful from a therapeutic viewpoint. The invention provides herewith a pharmaceutical composition comprising a nucleic acid, vector, host cell, or proteinaceous substance according to the invention. In addition, such a pharmaceutical composition according to the invention is provided further comprising a nucleic acid encoding apoptin or a functional equivalent or fragment thereof or apoptin or a functional equivalent or fragment thereof.

Such a pharmaceutical composition is in particular provided for the induction of apoptosis, for example wherein said apoptosis is p53-independent, for the treatment of a disease where enhanced cell proliferation or decreased cell death is observed, as is in general the case when said disease comprises cancer or auto-immune disease. Herewith the invention provides a method for treating an individual carrying a disease where enhanced cell proliferation or decreased cell death is observed comprising treating said individual with a pharmaceutical composition according to the invention. In particular these compositions comprise a factor of an apoptosis pathway, which is specific for transformed cells. Therefore, these compositions are essential for new treatments, but also for diagnosis of

diseases related with aberrancies in the apoptotic process, such as cancer and auto-immune diseases.

In the field of diagnosis the invention provides a method for detecting the presence of cancer cells or cells that are cancer prone in a sample of cells comprising transfecting cells in said sample with a nucleic acid according or a vector to the invention culturing said cells and determining the percentage of apoptosis of cells in said sample. For example, we can conclude that the cellular localization of AAP-1 is different in tumorigenic/transformed cells in comparison to normal non-transformed cells. Furthermore, accumulation of AAP-1 in the nucleus correlates with apoptosis induction, whereas cytoplasmic localization correlates with cell viability and normal proliferative capacity. The invention thus provides a method for detecting the presence of cancer cells or cells that are cancer prone in a sample of cells comprising transfecting cells in said sample with a nucleic acid or a vector according to the invention and determining the intracellular localisation of a proteinaceous substance derived from said nucleic acid or vector in cells in said sample. In particular, the invention provides a wherein the presence of said proteinaceous substance in said cells is detected by immunostaining said cells with an antibody, such as with an immunofluorescence assay, or an other immunoassay known in the art. Preferably, said antibody comprises an antibody according to the invention.

Also, the invention provides a method for identifying a putative cancer-inducing agent, such as transforming genes or functional fragments thereof comprising submitting a sample of cells to said agent, for example by tranfection, or by merely providing the agent to the medium surrounding the cells, and detecting the presence of cancer cells or cells that are cancer prone in a sample of cells with a method according to the invention.

In addition, the invention provides a method to detect cancer-proneness of a sample of cells, and thereby to detect cancer-proneness of the individual from which those cells were sampled, comprising submitting said cells to a cancer-inducing agent, such as UV-light, and detecting the presence of cancer cells or cells that are cancer prone in a sample of cells with a method according to the invention.

The invention will be explained in more detail in the following detailed description which is not limiting the invention.

Experimental

We have used the yeast-2 hybrid system (Durfee et al., 1993) to identify apoptin-associating cellular compounds, which are essential in the induction of apoptosis. The used system is an in-vivo strategy to identify human proteins capable of physically associating with apoptin. It has been used to screen cDNA libraries for clones encoding proteins capable of binding to a protein of interest (Fields and Song, 1989, Yang et al., 1992). The invention provides a for example novel apoptin-associating protein, one of which is named apoptin-associating protein 1, abbreviated as AAP-1. The invention also provides a method for inducing apoptosis through interference with the function of this newly discovered AAP-1 protein or functional equivalents or fragments thereof and/or the induction of apoptosis by means of (over)expression of AAP-1 or related gene or functional equivalents or fragments thereof.

The invention also provides an anti-tumor therapy based on the interference with the function of AAP-1-like proteins and/or its (over)expression. AAP-1-like proteins are normally not very abundantly present in immortalized cell lines. Therefore, an aberrant high level of AAP-1-like proteins will result in the induction of the opposite process of cell transformation, namely apoptosis. The invention furthermore provides the mediator of apoptin-induced apoptosis, which is tumor-specific. The invention provides a therapy for cancer, auto-immune diseases or related diseases which is based on AAP-1-like proteins alone or in combination with apoptin and/or apoptin-like compounds.

Materials and Methods

Construction of pGBT9-VP3

For the construction of the bait plasmid, which enables the identification of apoptin-associating proteins by means of a yeast-two-hybrid system, plasmid pET-16b-VP3 (Noteborn, unpublished results) was treated with NdeI and BamHI. The 0.4 kb NdeI-BamHI DNA fragment was isolated from low-melting-point agarose.

GAL4-activation domain-tagged cDNA library

The expression vector pACT, containing the cDNAs from Epstein-Barr-virus-transformed human B cells fused to the GAL4 transcriptional activation domain, was used for detecting apoptin-associating proteins. The pACT c-DNA library is derived from the lambda-ACT cDNA library, as described by Durfee et al. 1993.

Bacterial and Yeast strains

The E.coli strain JM109 was the transformation recipient for the plasmid pGBT9 and pGBT-VP3. The bacterial strain electromax/DH10B was used for the transformation needed for the recovery of the apoptin-associating pACT-cDNAs, and was obtained from GIBCO-BRL, USA.

The yeast strain Y190 was used for screening the cDNA library, and all other transformations, which are part of the used yeast-two-hybrid system.

Media

For drug selections Luria Broth (LB) plates for E.coli were supplemented with ampicillin (50 microgram per ml). Yeast YPD and SC media were prepared as described by Rose et al. (1990).

Transformation of competent yeast strain Y190 with plasmids pGBT-VP3 and pACT-cDNA and screening for beta-galactosidase activity.

The yeast strain Y190 was made competent and transformed according to the methods described by Klebe et al. (1983). The yeast cells were first transformed with pGBT-VP3 and subsequently transformed with pACT-cDNA, and these transformed yeast cells were grown on histidine-minus plates, also lacking leucine and tryptophan.

Hybond-N filters were layed on yeast colonies, which were histidine-positive and allowed to wet completely. The filters were lifted and submerged in liquid nitrogen to permeabilize the yeast cells. The filters were thawed and layed with the colony side up on Whattman 3MM paper in a petridish with Z-buffer (Per liter: 16.1 gr $Na_2HPO_4.7H_2O$, 5.5 gr $NaH_2PO_4.H_2O$, 0.75 gr KCl and 0,246 gr $MgSO_4.7H_2O$,

pH 7.0) containing 0.27% beta-mercapto-ethanol and 1 mg/ml X-gal. The filters were incubated for at least 15 minutes or during night.

Recovery of plasmids from yeast

Total DNA from yeast cells, which were histidine- and beta-galactosidase-positive, was prepared by using the glusulase-alkaline lysis method as described by Hoffman and Winston (1987) and used to transform Electromax/DH10B bacteria via electroporation using a Bio-Rad GenePulser according the manufacturer's specifications.

Transformants were plated on LB media containing the antibiotic agent ampicillin.

Isolation of apoptin-associating pACT clones

By means of colony-filter assay the colonies were lysed and hybridized to a radioactive-labeled 17-mer oligomer, which is specific for pACT (see also section Sequence analysis). Plasmid DNA was isolated from the pACT-clones, and by means of XhoI digestion analysed for the presence of a cDNA insert.

Sequence analysis

The subclones containing the sequences encoding apoptin-associating proteins were sequenced using dideoxy NTPs according to the Sanger-method, which was performed by Eurogentec, Seraing, Belgium). The used sequencing primer was a pACT-specific 17-mer comprising of the DNA-sequence 5'-TACCACTACAATGGATG-3'.

The sequences of the apoptin-associating cDNAs were compared with known gene sequences from the EMBL/Genbank.

Construction of pMAL-AAP1 and pET22b-AAP1

For the construction of the protein overexpression plasmids, which enable the production and isolation of apoptin-associated protein, plasmids pMALTB and pET22b were used. Plasmid pMALTB is a derivative of pMAL-C2 (New England Biolabs) in which the factor Xa site has been replaced by a Trombin site. Plasmid

pMALTB was treated with BamHI and SalI and the ± 7.0 kb DNA fragment was isolated from an agarose/TBE gel (QIAGEN gel extraction kit). The AAP1 sequence encoding the complete open reading frame was obtained by a PCR reaction on pACT-AAP-1b with the forward primer:
5'AACGGGATCCGGCGGCATGGGCGACAAGAAGAGCCCGACC 3' and the reversed primer:
5'AAAAGTCGACTCAGAAAGATTCATCATTGACTGCTGACAT 3';
the ± 0.7 kb PCR fragment was digested with BamHI and SalI and isolated from an agarose/TBE gel (QIAGEN gel extraction kit). The final construct containing a fusion between the MBP gene and the AAP1 gene under the regulation of the IPTG inducible tac promoter was called pMAL-AAP1.

Plasmid pET22b (Novagen)was treated with NdeI and NotI and the ± 5.5 kb DNA fragment was isolated from an agarose/TBE gel (QIAGEN gel extraction kit). The AAP1 sequence encoding the open reading frame was obtained by a PCR reaction on pACT-AAP-1b with the forward primer:
5' GGGAATTCCATATGGGCGACAAGAAGAGCCCGACC 3' and the reversed primer:
5' AAGGAAGTACGCGGCCGCGAAAGATTCATCATTGACTGCTGACATGT 3';
the PCR product was treated with NdeI and NotI and the ± 0.7 kb fragment was isolated from an agarose/TBE gel (QIAGEN gel extraction kit). The final construct containing a fusion between the AAP1 gene and the (His)6-tail under the regulation of the IPTG inducible T7lac promoter was called pET22b-AAP1.

Both constructs were proven to be correct by restriction-enzyme analysis and DNA-sequencing according to the Sanger method (1977)

All cloning steps were essentially carried out as described by Maniatis et al. (1992).

Bacterial strains for overexpression of MBP-AAP1 and AAP1-(His)6

For protein production with the plasmid pMAL-AAP1 the E. coli strain B834(λDE3) was used and for the plasmid pET22b-AAP1 the E. coli strain BL21(DE3) was used. Both strains were obtained from Novagen.

Apoptin induces specifically apoptosis in transformed cells, such as cell lines derived from human tumors. To identify the essential compounds in this cell-transformation-specific and/or tumor-specific apoptosis pathway, a yeast genetic screen was carried out.

We have used a human cDNA library, which is based on the plasmid vector pACT containing the complete cDNA copies made from Epstein-Barr virus-transformed human B cells (Durfee et al., 1993).

The following examples are offered by way of illustration of the present invention, not limitation.

Example 1: Construction of a bait plasmid expressing a fusion gene product of GAL4-DNA-binding domain and apoptin

To examine the existence of apoptin-associating proteins in the human transformed/tumorigenic cDNA library, a so-called bait plasmid had to be constructed. To that end, the complete apoptin-encoding region, flanked by about 40 basepairs downstream from the apoptin gene, was cloned in the multiple cloning site of plasmid pGBT9.

The final construct, called pGBT-VP3, was analysed by restriction-enzyme analysis and sequencing of the fusion area between apoptin and the GAL4-DNA-binding domain.

Example 2: A gene(fragment) encoding an apoptin-associating protein is determined by transactivation of a GAL4-responsive promoter in yeast.

The apoptin gene is fused to the GAL4-DNA-binding domain of plasmid pGBT-VP3, whereas all cDNAs derived from the transformed human B cells are fused to the GAL4-activation domain of plasmid pACT. If one of the proteinaceous substances encoded by said cDNAs binds to apoptin, the GAL4-DNA-binding domain will be in the vicinity of the GAL4-activation domain resulting in the activation of the GAL4-responsive promoter, which regulates the reporter genes HIS3 and LacZ.

The yeast clones containing plasmid expressing apoptin and a plasmid expressing an apoptin-associating protein fragment can grow on a histidine-minus medium and will stain blue in a beta-galactosidase assay. Subsequently, the plasmid

with the cDNA insert encoding the apoptin-associating protein can be isolated and characterized.

Before we could do so, however, we have determined that transformation of yeast cells with pGBT-VP3 plasmid alone, or in combination with an empty pACT vector, did not result in the activation of the GAL4-responsive promoter.

Example 3: Identification of apoptin-associating proteins encoded by cDNAs derived from a human transformed B cell line.

We have found two yeast colonies, which upon transformation with pGBT-VP3 and pACT-cDNA was able to grow on a histidine-minus medium (also lacking leucine and tryptophan) and stained blue in a beta-galactosidase assay. These results indicate that the observed yeast colonies contain besides the bait plasmid pGBT-VP3 also a pACT plasmid encoding a potential apoptin-associating protein.

Plasmid DNA was isolated from the positive yeast colony, which was transformed in bacteria. By means of a filter-hybridization assay using a pACT-specific labeled DNA-probe, the clones containing pACT plasmid could be determined. Subsequently, pACT DNA was isolated and digested with restriction enzyme XhoI, which is indicative for the presence of a cDNA insert. Finally, the pACT plasmids containing a cDNA insert was sequenced by using the Sanger method (Sanger et al., 1977).

Example 4: Description of apoptin-associating proteins

The yeast genetic screen for apoptin-associating proteins resulted in the detection of two cDNA clones A and B comprising a single type of protein, namely a novel protein called apoptin-associating protein 1, abbreviated as AAP-1. The cDNA AAP-1-b harbors the complete open reading frame with an ATG-initiation codon, whereas the AAP-1-a cDNA sequence contains a partial AAP-1 open reading frame, which is completely homologous to the AAP-1-b DNA sequence.

The determined DNA sequence of the AAP-1-a and AAP-1-b cDNA clones are shown in Figures 1 and 2, respectively. The amino acid sequence, derived from the detected DNA sequence of clone AAP-1-b, which represents the complete AAP-1 a.a. sequence, is given in Figure 3.

Example 5: Construction of an expression vector for the identification of AAP-1 protein in mammalian cells.

To study whether the cloned cDNAs AAP-1-a and AAP-1-b indeed encode (apoptin-associating) protein products, we have carried out the following experiments.

The DNA plasmid pMT2SM contains the adenovirus 5 major late promoter (MLP) and the SV40 ori enabling high levels of expression of foreign genes in transformed mammalian cells, such as SV-40-transformed Cos cells. Furthermore, the pMT2SM vector contains a Myc-tag (amino acids: EQKLISEEDL) which is in frame with the foreign-gene product. This Myc-tag enables the recognition of e.g. apoptin-associating proteins by means of the Myc-tag-specific 9E10 antibody.

The pMT2SM vectors expressing Myc-tagged AAP-1-a or AAP-1-b cDNAs were constructed as follows. The pACT-AAP-1-a and pACT-AAP-1-b cDNA clones were digested with the restriction enzyme XhoI and the cDNA inserts were isolated. The expression vector pMT2SM was digested with XhoI and treated with calf intestine alkline phosphatase and ligated to the isolated AAP-1 cDNA inserts. By sequence analysis, the pMT2SM constructs containing the AAP-1-a or AAP-1-b cDNA in the correct orientation were identified.

The synthesis of Myc-tagged AAP-1 protein was analyzed by transfection of Cos cells with plasmid pMT2SM-AAP-1-a or pMT2SM-AAP-1-b. As negative control, Cos cells were mock-transfected. Two days after transfection, the cells were lysed and Western-blot analysis was carried out using the Myc-tag-specific antibody 9E10.

The Cos cells transfected with pMT2SM-AAP-1-a and pMT2SM-AAP-1-b were proven to synthesize a specific Myc-tagged AAP-1 product with the expected size of approximately 33 kDa (AAP-1-a) or 35 kDA (AAP-1-b). As expected, the lysates of the mock-transfected Cos cells did not contain a protein product reacting with the Myc-tag-specific antibodies.

These results indicate that we have been able to isolate cDNAs that are able to produce a protein product with the ability to associate to the apoptosis-inducing protein apoptin.

Example 6: Co-immunoprecipitation of Myc-tagged AAP-1 protein with apoptin in a transformed mammalian cell system.

Next, we have analysed the association of apoptin and the AAP-1 protein by means of co-immunoprecipitations using the Myc-tag-specific antibody 9E10. The 9E10 antibodies were shown not to bind directly to apoptin, which enables the use of 9E10 for carrying out co-immuno-precipitations with (myc-tagged) apoptin-associating proteins and apoptin.

To that end, Cos cells were co-transfected with plasmid pCMV-VP3 encoding apoptin and with plasmid pMT2SM-AAP-1-a. As a negative control, cells were transfected with pCMV-VP3 expressing apoptin and a plasmid pcDNA3.1.LacZ-myc/His -LacZ encoding the myc-tagged beta-galactosidase, which does not associate with apoptin.

Two days after transfection, the cells were lysed in a buffer consisting of 50 mM Tris (7.5), 250 mM NaCl, 5 mM EDTA, 0.1 % Triton X100, 1 mg/ml $Na_4P_2O_7$ and freshly added protease inhibitors such as PMSF, Trypsine-inhibitor, Leupeptine and Na_3VO_4. The specific proteins were immuno-precipitated as described by Noteborn et al. (1998) using the Myc-tag-specific antibodies 9E10, and analyzed by Western blotting.

Staining of the Western blot with 9E10 antibodies and 111.3 antibodies, which are specifically directed against myc-tag and apoptin,respectively, showed that the "total" cell lysates contained apoptin and the Myc-tagged AAP-1 protein or beta-galactosidase product. Immunoprecipitation of the Myc-tagged AAP-1 products was accompanied by the immuno-precipitation of apoptin product of 16 kDa. In contrast, immunoprecipitation of myc-tagged beta-galactosidase did not result in a significant co-precipitation of the Apoptin protein.

In total, three independent immunoprecipitation experiments were carried out, which all showed the associating ability of apoptin to the AAP-1 protein.

These results indicate that the novel determined AAP-1 protein is able to specifically associate with apoptin not only in the yeast background, but also in a mammalian transformed cellular system.

Example 7: Over-expression of the novel AAP-1 protein in human transformed cells induces the apoptotic process.

In addition, we have examined whether AAP-1 carries apoptotic activity. First, we have analysed the cellular localisation of the novel AAP-1 protein in human transformed cells. To that end, the human osteosarcoma-derived Saos-2 cells were transfected, as described by Danen-van Oorschot (1997), with plasmid pMT2SM-AAP-1-a or pMT2SM-AAP-1-b encoding the myc-tagged AAP-1-a or AAP-1-b protein, respectively.

By indirect immunofluorescence using the myc-tag-specific antibody 9E10 and DAPI, which stains the nuclear DNA, it was shown that both the partial and complete AAP-1 protein were present in the nucleus of the cell. Actually, it co-localizes with the chromatin/DNA structures.

Finally, we examined whether (over)-expression of both cDNAs encoding complete or partial AAP-1 protein results in induction of apoptosis. Four days after transfection, the majority of AAP-1-positive cells were aberrantly stained with DAPI, which is indicative for induction of apoptosis (Telford, 1992, Danen-van Oorschot, 1997).

Co-expression of apoptin and both AAP-1 proteins in human tumor cells, such as Saos-2 cells, results in a faster apoptotic process as expression of apoptin or AAP-1 protein alone. The results of the apoptotic activity of the complete AAP-1 protein are shown in Figure 4.

The fact that AAP-1 protein can induce apoptosis in p53-minus Saos-2 cells indicates that AAP-1 can induce p53-independent apoptosis. These results imply that AAP-1 can be used as anti-tumor agent in cases where other (chemo)therapeutic agents will fail. Furthermore, the finding that both apoptin and AAP-1 induce a p53-independent pathway indicates that AAP-1 fits in the apoptin-induced apoptotic pathway.

Example 8: Co-localization of Apoptin and AAP-1 in human tumor cells

To establish the possible co-localization of Apoptin and AAP-1 in transformed human cells, plasmids encoding Apoptin and AAP-1 were transfected in Saos-2 cells. Expression of AAP-1 and Apoptin was monitored by indirect immunofluorescence by means of a confocal-laser scanning microscopy with the use of specific antibodies

mAb myc 9E10 against the myc-tag on AAP-1 and pAb VP3-c against the C-terminus of Apoptin.

Cells co-transfected with a plasmid encoding AAP-1 and a plasmid encoding Apoptin expressed these proteins predominantly in the nucleus. Both, Apoptin and AAP-1 had granular structures and the above described characteristic structures. By means of confocal-laser scanning microscopy, partial co-localization of AAP-1 and Apoptin was clearly shown to occur in these nuclear structures.

In conclusion, we have identified an apoptin-associating protein, namely the novel AAP-1 protein, which is present in the nucleus and able to induce (p53-independent) apoptosis in human tumor cells. Furthermore, when AAP1 and apoptin are expressed in the same cell the two apoptosis inducing proteins are co-localizing in the nucleus of human tumor cells.

Example 9: AAP-1 localizes in human normal diploid cells in cytoplasmic structures.

Next, we have examined whether AAP-1 behaves similar in normal human diploid non-transformed cells as has been found for AAP-1 in human tumor cells.

To that end, human diploid VH10 fibroblasts (Danen-Van Oorschot, 1997) were transfected using Fugene according the protocol of the supplier (Roche, Almere, The Netherlands), with plasmid pMT2SM-AAP-1b encoding the myc-tagged complete AAP protein. In parallel, human tumor-derived Saos-2 cells were also transfected with plasmid pMT2SM-AAP-1b.

By indirect immunofluorescence using the myc-tag-specific antibody 9E10, it was shown that in normal diploid VH10 fibroblasts AAP-1 protein is located in the cytoplasm. As expected, in the human tumor Saos-2 cells AAP-1 is located in the nucleus.

Furthermore, we have examined the effect of co-expression of AAP-1 and apoptin in human VH10 fibroblasts on the cellular localization of AAP-1, as described for cells expressing AAP-1 alone. The immunofluorescence data show that both apoptin and AAP-1 are located in cytoplasmic structures. These findings indicate that AAP-1 and/or apoptin expression do not result in the nuclear localization of one or the other in normal (human) diploid cells.

Example 9: Co-localization of Apoptin and AAP-1 in human fibroblasts

To establish the possible co-localization of Apoptin and AAP-1 in non-transformed cells, plasmids encoding Apoptin and AAP-1 were transfected in VH10 cells. Expression of AAP-1 and Apoptin were monitored by indirect immunofluorescence by means of a confocal-laser scanning microscopy with the use of specific antibodies mAb myc 9E10 against the myc-tag on AAP-1 and pAb VP3-c against the C-terminus of Apoptin. Cells were screened for (co-) localization of Apoptin and AAP-1 and for induction of apoptosis by nuclear staining with DAPI.

Cells co-transfected with a plasmid encoding AAP-1 and a plasmid encoding Apoptin expressed these proteins predominantly in the cytoplasm. Apoptin and AAP-1 both had a thready and sometimes granular structure. By means of confocal-laser scanning microscopy, co-localization of AAP-1 and Apoptin was clearly shown to occur in these cytoplasmic structures. Similar cytoplasmic structures were observed with AAP-1, when expressed alone or vice versa when Apoptin was expressed alone.

Thus, in the presence or absence of Apoptin, AAP-1 has a cytoplasmic localization and thready-aggregated structures in non-transformed human fibroblasts.

In conclusion, we have identified an apoptin-associating protein, namely AAP-1, which is differentially located in human diploid non-transformed cells versus human tumorigenic cells. These results show that AAP-1 functions in a different way in normal diploid cells as in tumorigenic cells. Furthermore, when AAP1 and apoptin are expressed in the same normal non-transformed cell the two apoptosis inducing proteins are co-localizing in the cytoplasm.

Example 10: The effect on induction of covalent linkage of an SV40 large T antigen nuclear localization signal to the apoptin protein.

In the following experiments, we have examined whether expression of a chimeric protein consisting of apoptin and the nuclear localization signal of SV40 LT antigen (amino acids N-terminal-Proline-Proline-Lysine-Lysine-Lysine-Arginine-Lysine-Valine-C-terminal of SV40 large T antigen covalently linked to the N-terminus of Apoptin) results in the induction of apoptosis in non- and transformed human cells. The chimeric protein is called NLS-apoptin.

To that end, non-transformed VH10 human fibroblasts and transformed human osteosarcoma-derived Saos-2 cells (Danen-van Oorschot et al., 1997) were transfected with a plasmid encoding the chimeric protein NLS-apoptin. In transformed human cells, expression of NLS-apoptin resulted in the nuclear localization of apoptin and induction of apoptosis. Expression of NLS-Apoptin in normal human fibroblasts, however, resulted in the nuclear localization of apoptin, but not in induction of apoptosis. This indicates that "forcing" of transporting apoptin into the nucleus does not result in its apoptotic activity per se. Apoptin seems to require an additional tumor-related event.

Example 11: The effect of expression of NLS-apoptin on AAP-1 in normal human fibroblasts.

Next, we have examined whether the expression of NLS-apoptin can influence the cellular location of AAP-1 and/or its apoptotic activity in normal human fibroblasts. To that end, VH10 cells were co-transfected with plasmids encoding NLS-apoptin or AAP-1. By means of indirect-immunofluorescence and DAPI-staining using a fluorescence microscopy, it was clearly established that both NLS-apoptin and AAP-1 were located in the nucleus without inducing apoptosis. This indicates that "forcing" of transporting AAP-1 through NLS-apoptin into the nucleus does not result in AAP-1 apoptotic activity per se. AAP-1 like apoptin seems to require an additional tumor-related event to become apoptotic in the nucleus of transformed cells.

Example 12: AAP-1 does not induce apoptosis in human diploid non-transformed cells.

In the following experiments we have examined whether AAP-1 alone or in combination with apoptin is also able to induce apoptosis in human diploid non-transformed fibroblasts as has been observed for human tumorigenic/transformed cells.

To that end, VH10 cells were transfected with plasmid pMT2SM-AAP-1-b as described above. The transfected cells were analyzed by indirect immunofluorescence using the myc-tag-specific and/or apoptin-specific antibodies and DAPI-staining. DAPI stains intact DNA in a different way than apoptotic DNA (Telford et al., 1992).

The analysis clearly shows that VH10 fibroblasts containing AAP-1 protein alone or both AAP-1 and apoptin do not undergo apoptosis.

The obtained results show that apoptin-related proteins such as AAP-1 might behave in a different way in "healthy" cells in comparison to tumor cells.

Example 13: Diagnostic assay for cancer cells

Based on the present report, we can conclude that the cellular localization of AAP-1 is different in tumorigenic/transformed human cells in comparison to normal human non-transformed cells. Furthermore, accumulation of AAP-1 in the nucleus correlates with apoptosis induction, whereas cytoplasmic localization correlates with cell viability and normal proliferative capacity. Therefore, we are able to develop a diagnostic assay for the identification of (human) cancer cells versus normal "healthy" non-transformed cells.

The assay consists of transfecting "suspicious" (human) cells, for instance from human origin, with a plasmid encoding AAP-1, or infecting the cells with viral vectors expressing AAP-1. Subsequently, the cells will be examined, 1) for the ability to undergo apoptosis by the over-expressing AAP-1 gene and 2) for a shift in the localization of AAP-1 from the cytoplasm to the nucleus.

The intracellular localization of AAP-1 can be determined, using an immunofluorescence assay with monoclonal antibodies specific for AAP-1 and/or specific for a tag linked to AAP-1 such as the herein described myc-tag. If the percentage of apoptosis and/or the nuclear localization of AAP-1 in the analyzed cells expressing AAP-1 is significantly higher than in AAP-1-positive control "healthy" cells, one can conclude that the analyzed cells has become tumorigenic/transformed. As positive control known human tumorigenic cells will be used for expressing AAP-1.

Example 14: Co-expression of SV40 large T antigen and AAP-1 results in translocation of AAP-1 and induction of apoptosis.

We have examined the effect of expression of transforming genes on AAP-1-induced apoptosis in normal human cells derived from healthy individuals. To that end, human VH10 diploid fibroblasts were transiently co-transfected with plasmid

pMT2SM-AAP-1b encoding the complete AAP-1 protein and either plasmid pR-s884 encoding SV40 large T antigen, or the negative-control plasmid pCMV-neo (Noteborn and Zhang, 1998).

By indirect immunofluorescence, the cells were analyzed for AAP-1-induced apoptosis. The normal VH10 cells did not undergo apoptosis when AAP-1 was transfected with the negative-control plasmid. The results showed, as expected, that expression of AAP-1 is not able to induce apoptosis in normal human diploid cells, confirming the above mentioned data. However, normal diploid human fibroblasts expressing both AAP-1 and SV40 large T antigen underwent AAP-1-induced apoptosis.

The transition of normal human cells, from AAP-1-resistance to AAP-1-susceptibility, can probably be explained by the fact that the AAP-1 protein translocates from a cytoplasmic localization to a nuclear localization. This transition becomes apparent already 2 days after transfection of plasmids encoding the transforming protein SV40 large T antigen. One can conclude that an event takes place, in this example due to expression of a transforming product derived from a DNA-tumor virus, which results in the translocation of over-expressed AAP-1 from the cytoplasm to the nucleus, which is followed by induction of apoptosis.

Example 15: Diagnostic assay for cancer-inducing genes, agents and cancer-proness based on AAP-1-induced apoptosis.

Based on the present report, we are able to develop a diagnostic assay for the identification of cancer-inducing and/or transforming agents or genes.

A first type of assay consists of transfecting "normal" cells, for instance from human origin, with a plasmid encoding AAP-1, or infecting the cells with viral vectors expressing AAP-1, together with a plasmid encoding a putative transforming/cancer-inducing gene. Subsequently, the cells will be examined, 1) for the ability to undergo apoptosis by the over-expressing AAP-1 gene and 2) for a shift in the localization of AAP-1 from the cytoplasm to the nucleus.

The intracellular localization of AAP-1 can be determined, using an immunofluorescence assay with monoclonal antibodies specific for AAP-1 and/or specific for a tag linked to AAP-1 such as the herein described myc-tag. If the

percentage of apoptosis and/or the nuclear localization of AAP-1 in normal cells co-expressing AAP-1 and the putative transforming/cancer-inducing gene is significantly higher than in AAP-1-positive control cells expressing a control plasmid, one can conclude that the analyzed gene indeed has transforming/cancer-inducing activity.

A second example of a diagnostic test is based on the treatment of cultured normal diploid cells with a putative carcinogenic agent. The agent can be added, for instance, to the culture medium for various lengths of time. Subsequently, the cells are transfected with a plasmid encoding AAP-1. This approach can also be carried out by first transfecting/infecting the normal diploid cells, and then treating the cells with the agent to be tested.

The subsequent steps of the assay are the same as described for the in this section described first type of diagnostic assay. If the percentage of apoptosis and/or the nuclear localization of AAP-1 in normal cells expressing AAP-1 and the putative carcinogenic agent is significantly higher than in AAP-1-positive control cells expressing a control agent, one can conclude that the analyzed agent indeed has transforming/cancer-inducing activity.

A third example of a diagnostic test is based on the treatment of cultured normal diploid cells derived from a skin biopsy of the potential cancer-prone individual to be tested and cultured in suitable medium. Next, the cells are irradiated with UV and subsequently transfected with a plasmid encoding AAP-1, or infected with a viral vector expressing AAP-1, or the cells are first transfected/infected and then irradiated. In parallel, diploid cells from a normal healthy individual will be used as control.

The subsequent steps of the assay are the same as described for the in this section described first type of diagnostic assay. If after UV-treatment the percentage of apoptosis and/or the nuclear localization of AAP-1 in diploid cells derived from the potential cancer-prone individual is significantly higher than in UV-treated AAP-1-positive control cells, one can conclude that the analyzed individual is cancer-prone.

Example 16: AAP-1 and YY1 associate with each other in transformed cells

The mouse homologue of AAP-1, RYBP (Garcia et al., 1999) can interact with YY1. YY1 can activate or repress the transcription of cellular and viral proteins.

Protein-protein interactions can influence the activity of YY1. AAP-1 might interact with YY1. The interaction between AAP-1 and YY1 could influence the activity of YY1, resulting in transcriptional repression or activation. Transcription of genes could be important for the apoptotic activity of Apoptin and/or AAP-1.

To establish whether AAP-1 forms a complex with YY1 in vitro, plasmids encoding AAP-1 and YY1 (pCMV-HAVY-1; Kind gift of Dr Yang Shi, Harvard Medical School, Boston, USA) were co-transfected in Cos-cells. As negative control, YY-1 was co-expressed with LacZ. The cells were lysed and an immunoprecipitation assay was performed with the use of specific antibodies against the myc-tag of AAP-1 (or LacZ), anti-myc 9E10, and against YY1, anti-YY1. The resulting complexes were analyzed by Western-blotting techniques. In the cell-lysates, AAP-1 and/or YY1 and/or LacZ were detected, as expected.

In cells transfected with AAP-1 and YY1 it was clearly visible that when we performed an immunoprecipitation with antibodies specific for myc-tagged AAP-1, both AAP-1 and YY1 were detectable. The blot showed a band at 32 kD (AAP-1) and a band at 67 kD (YY1). Additionally, we performed an immunoprecipitation using specific antibodies for YY1. Again, both YY1 and AAP-1 were detectable on the Western blot.

In cells transfected with a plasmid expressing only AAP-1, only AAP-1 could be detected. Cells transfected with a plasmid encoding YY1 showed expression of YY1, AAP-1 was not detected. To rule out a-specific binding of AAP-1 with YY1, cells were transfected with AAP-1 and LacZ and immunoprecipitations were carried out with antibodies specific for LacZ. Although, LacZ protein was clearly visible on the Western Blot, no AAP-1 protein was visible.

Therefore, the obtained results show that AAP-1 specifically binds to the transcription-related factor YY1.

Example 17: Production and isolation of MBP-AAP1 and AAP1-(His)6

To examine the possibility of MBP-AAP1 and AAP1-(His)6 fusionprotein production the AAP1 nucleic acid coding for the open reading frame was cloned in the protein overexpression cassettes pMALTB and pET22b. Inducing the E.coli cells

according to the manufactures instructions (Novagen) leads to the production of soluble fusionprotein.

Isolation of the MBP-AAP1 fusion protein via affinity chromatography (amylose resin, New England Biolabs) and ion exchange chromatography (High-S, Biorad) leads to a 90 to 95 % pure, full length protein. Size exclusion chromatography (Pharmacia Superose 6 HR 10/30) of this protein shows that a substantial part of the MBP-AAP1 preparation appears to behave as one distinct species, a homotri- or tetramer. This finding would suggest that recombinant AAP1 (in the form of a MBP fusion protein) is capable of assuming a correct fold and is likely to be biologically active.

Isolation of the AAP1-(His)6 protein with metal affinity chromatography (Ni^{2+}-NTA, QIAGEN) leads to a ± 80 % pure protein batch.

In conclusion, fusion of AAP1 to MBP results in properly folded, soluble AAP1 which is likely to be biologically active.

Example 18: Production of polyclonal antibodies directed against AAP-1 proteins.

For the production of polyclonal antibodies against AAP-1 proteins a putative immunogenic peptide was synthesized (AAP-1 peptide consists of the amino acids N/terminus-CTKTSETNHTSRPRLK-C/terminus; EuroGentec SA, Belgium). Subsequently, rabbits were injected with the specific peptides according the standard procedures of the manufacturer.

The serum derived from the rabbits injected with the AAP-1 peptide was shown to be specific for the above described AAP-1 products by means of ELISA and Western-blot assays. These results imply that we have generated specific antibodies, which can be used for detecting the Apoptin-associating protein AAP-1.

In conclusion, we have provided evidence that interference of specific factors with the function of AAP-1 proteins results in induction of apoptosis.
Therapies based on induction of (p53-independent) apoptosis are possible utilising the interference with the function of AAP-1 proteins. An example of such an interfering factor is apoptin. Another CAV-derived protein, which is known to induce apoptosis and also known to enhance apoptin activity is VP2 (Noteborn et al., 1997).

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporate by reference.

The invention now having been fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.